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Original Article

An Alkaline Phosphatase Reporter Gene Assay for Induction of CYP3A4 *In Vitro*

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Abstract

CYP3A4 probably has the broadest catalytic activity of any cytochrome P450. It is a crucial task to test new drug candidates in a reliable system for their ability to induce expression of this enzyme. Firstly, a total of 300 bp core distal enhancer of CYP3A4 XREM region (-7972/-7673) were amplified from human genomic DNA. The PCR product was then ligated into a human secretory alkaline phosphatase cDNA-containing reporter vector (pSEAP2-1) creating pX-SEAP2 plasmid. Secondly, 1143 bp of the CYP3A4 proximal promoter region (-1203/-61) was amplified from the genomic DNA and then ligated into pX-SEAP2 plasmid DNA (between XREM and alkaline phosphatase gene), creating pXP-SEAP2 plasmid. Reporter constructs were then co-transfected with an hPXR expression vector into human liver and intestinal cells in culture. Xenobiotic modulation of CYP3A4 promoter activity was determined by chemiluminescent secretory alkaline phosphatase assay. Significant CYP3A4 induction at the transcriptional level using three different cell lines and four classical CYP3A4 inducers was observed. Transfection of reporter constructs in HepG2, HuH7 and Caco-2 cells, in general, produced similar pattern of induction by the same drugs with the exception ofphenobarbital. The results suggest that, carefully designed reporter gene systems can provide a useful in vitro approach for characterization of possible CYP3A4 inducers.

Keywords: Cytochrome P450; *CYP3A4* promoter; CYP3A4 induction; XREM region; Reporter gene assay.

Introduction

Human CYP3A4 is usually the major cytochrome P450 enzyme in the human liver and is known to metabolise a large variety of xenobiotics and endogenous biochemicals (1). Approximately half of the therapeutic drugs currently on the market are substrates. The ability of a single protein to catalyse so many reactions has been confirmed in studies with recombinant proteins (2). Moreover, intestinal CYP3A4 expression accounts for significant first pass metabolism of orally administered medication. It is now known that CYP3A4 is highly inducible and this may account for a major mechanism in known cases of drug interactions. It is also plausible that CYP3A4 induction may be a key determinant of inter-individual variation in susceptibility to chemical toxicants. The

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abundance of CYP3A4 in the human liver, the large number of substrates of diverse chemical structures it metabolises, the multiple metabolic pathways involved and its inducibility are all reasons suggesting that CYP3A4 is one of the most interesting and clinically significant CYP enzymes to be studied (1).

CYP3A4 has considerable relevance for personalised medicine, carcinogenesis risk associated with exposure to procarcinogens and other xenobiotics (3). CYP3A4 is considered the most important and is the most extensively studied member of the CYP3A subfamily. CYP3A4 activities in liver biopsies vary up to 40-fold in the population (4, 5, 6). However, the variability measured *in vivo* is usually much smaller (five fold) (7). This variation is generally assumed to contribute to harmful drug interactions frequently encountered in development and application of drugs that are CYP3A4 substrates.

Because CYP3A4 has a significant role in the elimination of a large number of drugs and possible induction-based interactions, it is important to test new drug candidates in a reliable system for their ability to induce expression of this enzyme. This kind of assessment seems crucial in alleviating the liability associated with these types of drug interactions that lead to either a lack of efficacy or toxicity. Extrapolation of information obtained from animal data has been problematic due to marked species differences that have been demonstrated in the response of CYP3A family to inducers (8). Therefore, a practical approach to obtain reliable data to evaluate the ability of any drug to induce CYP3A4 expression is considered to be vital in the process of a new drug development. Several recent studies have used different reporter gene assay approaches that may not be afforded in an ordinary lab (9, 10, 11).

In the present study, a simple *in vitro* alkaline phosphatase-based reporter gene system has been constructed to evaluate CYP3A4 induction at the transcriptional level using three different cell lines and four classical CYP3A4 inducers.

Experimental

Chemicals and reagents

All chemicals and reagents used in the experimental work were of molecular biology grade purchased from Sigma–Aldrich (Dorset, UK) unless specified. FuGENE 6 transfection reagent was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). All restriction enzymes and T4 DNA ligase were supplied by Promega Corporation (Madison, WI, USA).

Reporter gene constructs

A total of 300 bp of the *CYP3A4* XREM (xenobiotic responsive enhancer module) region (-7972/-7673) was amplified from human genomic DNA using forward (5′-**cggacgcgt**-ACTTCATGCAAAAATGCTGG-3′) and reverse (5′-**ccgctcgag**-GTTCTTGTCAGAAGTTCAGC-3′) primers that created *MluI* and *XhoI* restriction enzyme digestion sites, respectively (designated in bold) in the PCR products. The digested and purified PCR product was then ligated into *MluI* plus *XhoI* digested and purified promoter-less plasmid DNA containing a human secretory alkaline phosphatase cDNA reporter vector (pSEAP2-1; Basic, BD Bioscience, Oxford, UK). The recombinant XREM-SEAP plasmid (designated pX-SEAP2, Figure 1) was transfected into *E*. coli TOP10F' (Invitrogen, Paisley, UK) cells and transformed colonies isolated. 1143 bp of the *CYP3A4* proximal promoter region (-1203/-61) was amplified from the genomic DNA using forward (5′-**cccaagctt**-GACCATGCCCCATCATTGC-3′) and reverse (5′-**ccggaattc**-TGCTGGGCTATGTGCATGGAGC-3′) primers, creating *HindIII* and *EcoRI* restriction enzyme digestion sites, respectively in the PCR products. The standard numbering system designates the first nucleotide of the ATG translation start site as position +1 and the nucleotide before it as position -1. The main *CYP3A4* transcription start site is at -104 of wild-type (*CYP3A4*1A*). The digested and purified PCR products were then ligated into *HindIII* plus *EcoRI* digested pX-SEAP2 plasmid DNA, creating pXP-SEAP2 plasmid (Figure 1) which was transfected into *E*. coli TOP10F' cells. The authenticity of the plasmids was confirmed by re-digestion with relevant restriction enzymes, PCR of the insert region and sequencing of the

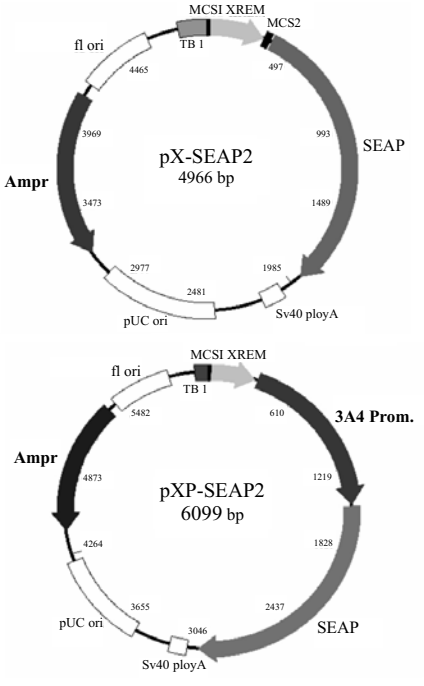

Figure 1. Map of constructed reporter vectors used in assessment of CYP3A4 induction. **Ampr**, ampicillin resistance gene; **f1 ori**, origin of replication of the filamentous phage f1; **MCS**, multiple cloning site; **pUC ori**, origin of replication; **SEAP**, cDNA for human secreted alkaline phosphatase; **SV40 Poly A**, simian virus 40 late mRNA polyadenylation signal; **TB**, transcription blocker (composed of a synthetic polyadenylation site and a transcription pause site from the human α2 globin gene); **XREM**, CYP3A4 distal xenobiotic responsive enhancer module; **3A4 Prom**, 1141 bp proximal promoter region of cytochrome P450 3A4.

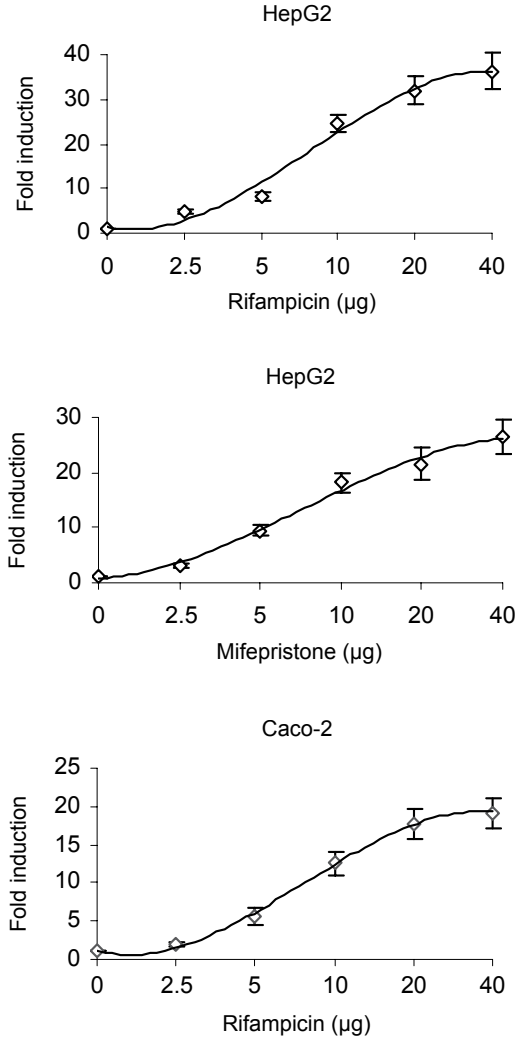

Figure 2. Dose response curves for rifampicin and mifepristone as inducers of pXP-SEAP2 expression following co-transfection with hPXR into HepG2 or Caco-2 cells. Fold inductions were calculated as the amount of relative light units produced by the drug in 0.1% DMSO divided by the amount of relative light units produce by 0.1% DMSO as solvent. Data represent the mean ± SEM, n=6.

PCR product using the constructed plasmids as template. The human pregnane X receptor expression plasmid (pSG5-hPXRΔATG) was a gift from Dr Steven Kliewer (GlaxoSmithKline Ltd). This expression vector was generated by PCR amplification and subcloning of 1.3 kb of hPXR cDNA, modified at the first codon (CTG →ATG) for improved protein expression, into the expression vector pSG5 (12). The positive control alkaline phosphatase expression vector (pCMV-cSPAP) was a gift from Dr Steve Hood and colleagues (GlaxoSmithKline).

Cell culture and DNA transfection

Cells were routinely grown in minimal essential medium with Earl's salts supplemented with 10% foetal calf serum, glutamine, gentamicin and non-essential amino acids (Invitrogen, Paisley, UK). HepG2 (a human hepatocyte carcinoma cell line) and Caco-2 (a human colon adenocarcinoma cell line) were obtained as low passage frozen cultures from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The HuH7 human hepatocellular carcinoma cell line (13) was also a gift from Dr Steve Hood. Following trypsinization, cells were diluted with growth medium to a concentration of 2×10^5 cell/ml and 120 µl of cell suspensions were seeded into each well of a 96-well plate (Nunc International, Leicester, UK). Plates were then incubated at 37°C for 48 h in a humidified container. FuGENE 6-mediated DNA co-transfections, using pXP-SEAP2

plasmid DNA containing *CYP3A4* promoter and pSG5-hPXRΔATG plasmid DNA, were performed as described by Goodwin *et al* (14). Transfections were allowed to proceed for 5 h in medium from which the serum had been omitted. Cells were then cultured for an additional 60 h in fresh serum-containing medium in the presence or absence of classical CYP3A4 inducers (14).

Determination of secreted alkaline phosphatase activity

A total of 15 µl samples of culture medium from transfected and control wells were transferred into an Optiplate polystyrene 96-well plate (Packard, Pangbourne, UK) and SEAP activity measured using the AuroraTM AP Chemiluminescent Reporter Gene assay system (ICN Biochemicals, Inc., Costa Mesa, USA). An automated LumiCountTM (Packard) plate reader was used to analyse the chemiluminescence output.

Data analysis

The change in SEAP activity measured before and 60 h after addition of xenobiotic was calculated for each reporter construct to allow for variation in cell seeding, transfection efficiency and cytotoxicity or cell proliferative effects of xenobiotics that might otherwise cause anomalous results. Activation (induction) and the statistical significance relative to the DMSO (solvent for drugs) control were tested as described (15).

Results and Discussion

Although detailed dose-response measurements were beyond the main scope of this project some experiments were performed to check pXP-SEAP2 reporter construct activity and confirm the effective range of drug concentrations. As shown in Figure 2, significant induction of CYP3A4 promoter commenced at 5 µM rifampicin and mifepristone, and a maximum effect was achieved around 40 µM. It is shown that at 10 µM rifampicin and mifepristone could produce significant induction of the promoter activity. These are the same concentration as used by Goodwin *et al* (14). For clotrimazole and Phenobarbital, 10 µM and 1 mM, were used respectively by the same authors.

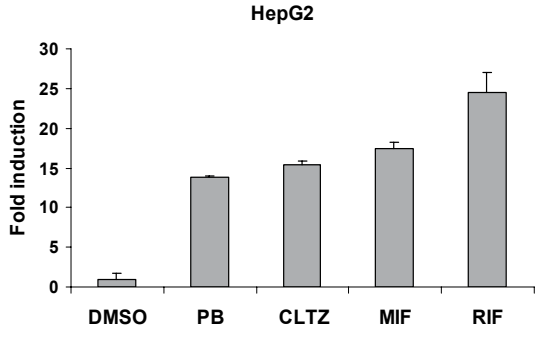

Figure 3. Induction profile of reporter construct containing the CYP3A4 promoter in the HepG2 cell line. Cells were co-transfected with pSG5-hPXR plasmid. Phenobarbital (1 mM); Clotrimazole (10 µM); Mifepristone (10 µM); Rifampicin (10 µM). Fold inductions were calculated as the amount of relative light units produced by the drug in 0.1% DMSO divided by the amount of relative light units produce by 0.1% DMSO as solvent. Data represent the mean ± SEM, n=6.

Results obtained from activation profile in HepG2 cell line (Figure 3) clearly demonstrate the ability of the reporter construct to detect CYP3A4 inducers in vitro. Similar results were also obtained in HuH7 and Caco-2 cell lines (Figures 4, 5). Transfection of reporter constructs in HepG2, HuH7 and Caco-2 cells, in general, produced similar pattern of induction by the same drugs. However, the only exception was phenobarbital which produced more induction in HuH7 cells compared to clotrimazole and mifepristone, and was the most potent inducer in Caco-2 cell line.

Based on previous experiments (16) and published literature, an in vitro reporter gene system was designed and constructed to assay induction of human CYP3A4 by xenobiotics. This system includes a distal enhancer region (XREM) of the gene which makes it very similar to natural hepatic environment. The results obtained for well-known CYP3A4 inducers in HepG2 cells are consistent with previously published reports (14) which indicate reliability of the system. There is no extensive information published regarding the HuH7 cell line and expression of cytochrome P450 enzymes except a report by Pascussi *et al* (17) indicating the expression of CYP3A4 by typical PXR activators rifampicin, clotrimazole and mifepristone. However, study of CYP3A4 promoter-dependant reporter gene construct expression in another hepatoma cell

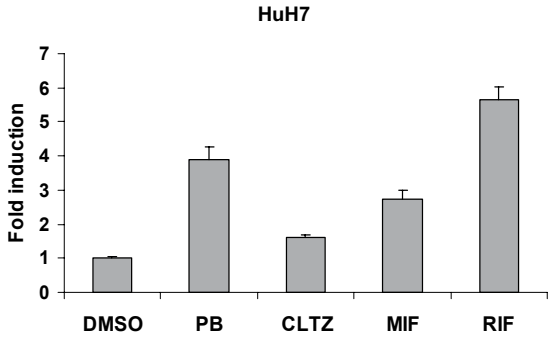

Figure 4. Induction profile of reporter construct containing the CYP3A4 promoter in the HuH7 cell line. Cells were co-transfected with pSG5-hPXR plasmid. Phenobarbital (1 mM); Clotrimazole (10 μM); Mifepristone (10 μM); Rifampicin (10 μM). Fold inductions were calculated as the amount of relative light units produced by the drug in 0.1% DMSO divided by the amount of relative light units produce by 0.1% DMSO as solvent. Data represent the mean ± SEM, n=6.

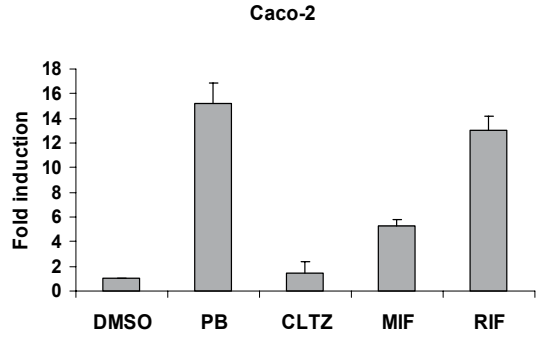

Figure 5. Induction profile of reporter construct containing the CYP3A4 promoter in the Caco-2 cell line. Cells were co-transfected with pSG5-hPXR plasmid. Phenobarbital (1 mM); Clotrimazole (10 μM); Mifepristone (10 μM); Rifampicin (10 μM). Fold inductions were calculated as the amount of relative light units produced by the drug in 0.1% DMSO divided by the amount of relative light units produce by 0.1% DMSO as solvent. Data represent the mean ± SEM, n=6.

D_3 (18). However, since CYP3A4 expression can be modulated in Caco-2 cells by xenobiotic inducers of hepatic CYP3A4, tranfections studies similar to the hepatoma cell lines were also performed in Caco-2 cells. The results also were similar indicating the ability of the system to detect xenobiotic induction in any cell line with possibility of CYP3A4 expression. Also of note was that in Caco-2 cells phenobarbital produced higher activation of some promoters than rifampicin. Another difference was that with all the reporter constructs, phenobarbital produced a greater effect than mifepristone or clotrimazole. This could be explained by higher expression of the nuclear receptor CAR in HuH7 cells. However, as with HepG2 cells rifampicin was the most powerful inducer. The different drug induction patterns in the different hepatoma cell lines again indicates that drug metabolism and interaction studies using liver cancer cells may not be a good reflection of the events in normal hepatocytes *in vivo* (Hamilton *et al*, 2001). However, considering the slightly different activation profiles in different cell types, it can be deduced that these responses are depend on the cellular environment, the availability of the transcription factors governing the expression of a specific gene in a specific tissue and the presence of endogenous or xenobiotic ligands and their concentrations.

In conclusion, a carefully designed and constructed reporter gene system can be a very valuable tool in characterization and functional analysis of CYP3A4 inducers *in vitro*. In our system wild type promoter region was inserted into alkaline phosphatase reporter vector and the potent upstream XREM region was also included in the construct. Considering the report by Goodwin *et al* (14) regarding the importance of co-transfection with hPXR expression plasmid in the study of CYP3A4 transcriptional activation, the experiments were performed in the presence of the hPXR expression vector pSG5-hPXR. It is clearly shown here that this system is applicable in any available cell line that expresses CYP3A4.

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